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Chemiluminescence is a rapid and sensitive method to assess phosphatidylcholine oxidation

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Abstract

The aim of this work was to test the suitability of chemiluminescence (CL) and isothermal microcalorimetry as simple, sensitive, and direct methods for the estimation of oxidation status of phosphatidylcholine in raw material and in lipid-based formulations. Such methods would avoid extensive sample preparation, allowing their use during early development work. Soya phosphatidylcholine (S-PC) was dissolved in medium-chain monoacylglycerol (MCM) and stored at 40°C in air and under nitrogen. Oxidative status was measured at different times by thiobarbituric acid value, iodometry, UV spectroscopy, high performance liquid chromatography (HPLC)-evaporative light scattering (ELS)-UV detection, isothermal microcalorimetry, and direct chemiluminescence. Oxidation parameters were analyzed in terms of the mechanism of oxidation. Data from the various methods were also found to be well correlated with that obtained from direct CL measurements. Induction times of about 21 days were found for a 50/50 w/w S-PC/MCM sample stored in air. MCM was found to have a protective effect on S-PC. Results indicate that both isothermal microcalorimetry and direct CL techniques can be used for estimation of the oxidation levels in phosphatidylcholines and should be applicable to phospholipids in general. MCM is a novel solvent for this purpose. CL measurements have the additional advantages of being rapid and convenient. Specifics of method development have been discussed.

Keywords: Phospholipid oxidation assay; Lipid oxidation assay; Monoglyceride; Thiobarbituric acid value; Iodometry; Evaporative light scattering detection; UV spectroscopy; Isothermal microcalorimetry; Chemiluminescence; Phosphatidylcholine oxidation

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Initiation	Substrate RH $\longrightarrow$ R$^\bullet$	(1)
Propagation	R$^\bullet$ + O_2 $\longrightarrow$ ROO$^\bullet$	(2)
	ROO$^\bullet$ + RH $\longrightarrow$ ROOH + R$^\bullet$	(3)
Termination	R$^\bullet$ + R$^\bullet$ $\longrightarrow$ R_2	(4)
	ROO$^\bullet$ + R$^\bullet$ $\longrightarrow$ ROOR	(5)
	ROO$^\bullet$ + ROO$^\bullet$ $\longrightarrow$ RHC=O* + O_2 + ROH	(6)

Scheme 1. $^\bullet$ Indicates radical; * indicates molecule in excited state.

1. Introduction

Lipids are used in a variety of pharmaceutical compositions in the form of emulsions, liposomal formulations, depots, suppositories etc. (Banakar and Speake, 1990). New drug delivery concepts based on lipids have been reported, e.g. a cholesterol/phosphatidylcholine matrix (Khan et al., 1993), solid lipid microparticles for parenteral use (Siekmann and Westesen, 1992; Schwarz et al., 1994), lecithin microcapsules (Fukumori et al., 1994), cholesteryl acetate nanoparticles (Sjöström et al., 1995), and a matrix based on a mixture of polar and nonpolar lipids called the Biosomes developed by our group (Bohlinder et al., 1994; Carlsson et al., 1994). All lipid based systems consisting of unsaturated lipids are susceptible to degradation by oxidation. The formulator has two ways of getting around this problem: using saturated lipids where possible, e.g. hydrogenated soya phosphatidylcholine (Khan et al., 1993) or through the addition of fat and/or water soluble antioxidants. Air tight packaging, and chelators such as EDTA also help to reduce this problem. However, measurement of the oxidation state of lipids is an important requirement during the development of such lipid-based systems for drug delivery. The aim of this work was to develop a simple and rapid method to evaluate oxidation status of (phospho)lipids for formulation work. The evolution of heat and light during the oxidation process were to be the measured parameters.

1.1. Review of lipid oxidation and measurement methods

Lipid oxidation is an important phenomena not only because of its contribution to deterioration of foods and fats, but also because of its pathological effects and its suspected contributions to atherosclerosis, cancer and ageing (Halliwell and Gutteridge (1990) and references therein). The mechanisms of oxidation and analytical methods for its detection and measurement have been the subject of extensive research. Oxidative degradation of lipids proceeds through a free radical chain mechanism which can be divided into three steps: initiation, propagation and termination. A simplified example is shown in reaction Scheme 1.

Once initiated (by loss of hydrogen radical in the presence of trace metals, heat or light), the alkyl radical of lipid fatty acid chain can react with atmospheric oxygen to produce an alkyl-peroxy radical (reaction 2 in Scheme 1). The peroxyl radical can abstract an allylic hydrogen from another unsaturated acyl chain to produce a lipid hydroperoxide and generate a new alkyl radical (reaction 3 in Scheme 1); the new radical reacts with oxygen to continue the propagation step as long as sufficient oxygen is available. Lipid hydroperoxide is a fundamental primary product of oxidation. Decomposition of lipid hydroperoxides constitutes a complicated set of reaction pathways leading to a multitude of volatile and non-volatile compounds. Hydroperoxides can react again with oxygen to form products such as epoxy-, keto-, di-, cyclic-, and bicyclic-hydroperoxides; the secondary hydroperoxides on further breakdown lead to the formation of a number of other prod-

ucts including malondialdehyde. The termination reaction in lipid oxidation can occur in three major ways (reactions 4–6 in Scheme 1). All of these lead to non-radical products including aldehydes, ketones, alcohols, hydrocarbons, esters, furans and lactones (Frankel, 1984).

As is apparent from the above scheme, the process of oxidation leads to a number of intermediate and final products; methods for assessing oxidation are therefore based on detecting some characteristic component. Among the common methods are iodometry to measure total hydroperoxides (also called peroxide value) and anisidine value (p-AV) as a measure of aldehydic components. Iodometry is time-consuming and requires a skilful analyst to obtain good precision. It lacks specificity and is susceptible to background noise due to oxidants and to drifts caused by dissolved oxygen; cyclic peroxides are not measured (Pryor and Castle, 1984). Determination of anisidine value requires absolutely dry samples which have to be dissolved in hexane or iso-octane. The actual value obtained depends not only on the amount of aldehydic components but also on their structure. Finally, the method is not particularly suited for phospholipids since they are highly hygroscopic in general and not soluble in hexane or iso-octane. The above two are standard methods in the fats/oils literature and are included in the AOAC Official methods of analysis (AOAC, 1990; Gunstone et al., 1986).

The colorimetric reaction of thiobarbituric acid with malondialdehyde (MDA) to obtain a thiobarbituric acid value (thiobarbituric acid reacting substances, TBARS) is widely used, especially in the biochemical literature, but is subject to criticism. MDA arises in fatty acid chains containing three or more double bonds only; thus it underestimates the total hydroperoxides content. Furthermore, the method does not specifically measure MDA only. Metal chelating agents can interfere in the reaction. The method has been recently reviewed by Draper and Hadley (1990).

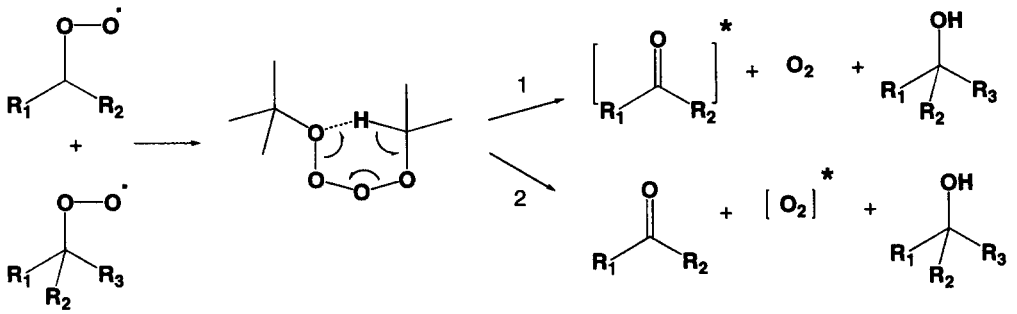
Oxidation of unsaturated fatty acids is also accompanied by an increase in UV absorption at 233 nm due to conjugated dienes and around 270–280 nm due to conjugated trienes. Maxima at 233 nm indicates oxidation of fatty acids with two or more double bonds while maxima at the higher wavelength arises due to fatty acids with three or more double bonds. Diene conjugation occurs at an early stage and 233 nm is therefore a useful wavelength to monitor (Klein, 1970; Pryor and Castle, 1984; Halliwell and Gutteridge, 1990). However, formation of secondary products leads to a loss of the conjugated diene structure.

Gas chromatography (GC) can be used to detect (by hydrolysis and methylester formation of free fatty acids) the loss of unsaturated fatty acids due to oxidation. Volatiles (aldehydes, ketones, alcohols etc.) can also be detected by (head space) GC (Frankel et al., 1989). Finally, HPLC based methods are reported wherein the hydroperoxides are detected by a post-column reaction system; the microperoxidase-catalyzed luminal-hydroperoxide reaction is used to induce chemiluminescence (Belghmi et al., 1988; Yamamoto et al., 1987, 1990).

1.2. Chemiluminescence (CL)

CL in lipid oxidation is considered to mainly arise in the termination stage. To produce CL, a reaction must lead to a product in an excited state and the product must be able to emit a photon rather than be deactivated in radiationless process. It is generally accepted that the CL emitter is an excited carbonyl; however, the source of this carbonyl is debated. The most accepted theory is based on the Russel mechanism. The bimolecular alkyl-peroxy radical termination step (reaction 6 in Scheme 1) occurs via the unstable tetraoxide intermediate shown in Scheme 2, and requires at least one of the terminating radicals to be secondary. The reaction is sufficiently exothermic (approximately 460 kJ/mol) to produce an excited carbonyl (triplet state in ketone), an alcohol, and molecular oxygen (reaction pathway 1 in Scheme 2).

A number of alternative mechanisms for the origin of CL from hydroperoxides have also been proposed (Gundermann and McCapra, 1987). In the absence of oxygen, the alkyl-peroxy radicals are generated from the decomposition of hydroperoxides already present in the sample.



Scheme 2.

Based on the above mechanism, CL emanating from a lipid sample in an inert atmosphere can be taken to be a measure of its peroxide content (Mendenhall, 1990).

CL is used to measure peroxides usually by the post column luminal-hydroperoxide reaction as mentioned above. Other researchers have measured the CL intensity more directly in luminometers etc., using energy transfer agents such as sodium hypochlorite (Yamamoto et al. (1985) for tetralyl hydroperoxide; Burkow et al. (1992) for cod liver oil) or potassium tert-butoxide (Neeman and Joseph, 1985) to increase the CL signal-to-noise ratio. Burkow et al. (1992) found good correlations between the CL counts from oxidized cod liver oil to parameters such as peroxide value, *p*-AV, TBARS, Kreis rancidity index, UV absorption at 233 nm, and sensoric score. However, the method is highly dependent on experimental conditions employed, with the preparation of emulsion between the sample oil dissolved in tert-butanol and NaOCl dissolved in 0.1 M NaOH being the critical step. Any antioxidants present in the sample were found to interfere with the result. Murphy and Sies (1990) have reviewed CL evolution in complex biological systems such as cells, tissues, organs etc. They summarized that correlations between CL and parameters such as oxygen uptake, TBARS, or conjugated dienes were not straightforward, depending on the system and stage of oxidation.

Direct measurement of peroxides by CL has also been made by Usuki et al. (1979) and Miyazawa et al. (1994) on soya bean oil, and by Miyagawa et al. (1991) on oil from frying. In all the cases, a good correlation was found between the CL counts and the various oxidation parameters mentioned above. In the work reported by Miyazawa et al. (1994), measurements were carried out by heating the sample at 100°C for 500 s on a stainless-steel plate. Interference by antioxidants (tocopherols) present was negligible. While good correlations were obtained, the oxidation status of oil heated in air on a plate containing iron will change during the measurement process (Pei et al., 1989).

1.3. Thermal analysis and oxidation measurements

Thermal parameters such as heat flux are directly linked to the oxidation of lipids. This fact has been utilized in detecting the susceptibility of lipids to oxidation by differential scanning calorimetry (DSC) (Pereira and Das, 1990; Yamazaki et al., 1980). Thermal analysis is however not a common technique in this area and references on the use of isothermal microcalorimetry for this purpose are few to non-existent. Raemy et al. (1987) used an isothermal heat flux calorimeter to study the oxidation process of fatty acid methyl esters, food oils, linseed oil etc. Samples were introduced under argon and oxidation induced by keeping the samples at temperatures between 80 and 160°C under an oxygen flow. Oxidation induction times were measured for samples exhibiting different thermal behavior, including unsaturation, and antioxidants in order to show the suitability of this technique. Similarly Kasprzycka-Guttman and Odenziak (1994) ob-

tained oxidation induction times for edible oils as a function of antioxidant type and concentration by isothermal calorimetry. Quantification of oxidation levels was not attempted in these references.

1.4. Aim of this work

While there are a number of methods available to assess oxidation, each of the methods has its limitations or complications. During the course of development of the Biosome system (Bohlinder et al., 1994), a rapid and convenient method for detection of oxidation was sought. None of the methods discussed above, except perhaps that based on UV spectroscopy, is really convenient and sensitive as a screening tool during development work. Direct measurement of hydroperoxides by CL however does seem to satisfy both these criteria.

A survey of the literature however showed no reports on the direct measurement of oxidation of phospholipids by CL or by isothermal microcalorimetry. The objective of this work was therefore to develop and evaluate these methods for assessment of (soya) phosphatidylcholine oxidation. Among the other methods that were used to correlate the result are TBARS, iodometry, UV abs. 233 and 270 nm, and HPLC with evaporative light scattering (ELS)-UV detection. The TBARS and iodometry methods were adapted directly from the literature, the UV absorbance and HPLC-ELS-UV detection methods were modified in-house, while the microcalorimetry and CL methods were developed for this study.

The samples analyzed in this work are mixtures of soya phosphatidylcholine (S-PC) and medium-chain monoglycerol (MCM) (weight ratios 50/50 and 30/70 S-PC/MCM). MCM is an inert component as far as oxidation is concerned, and may be considered as a solvent for S-PC in this case. It eases the handling of S-PC which, in dry anhydrous form, is highly hygroscopic and sticky. Dissolution of S-PC in MCM produces a homogeneous paste with low hygroscopicity, and a viscosity which increases with S-PC content. It was also proposed to test the hypothesis that MCM can protect S-PC from oxidation.

For the sake of completeness it must be mentioned that CL has been extensively used in the study of oxidation and autooxidation of plant and animal tissues, hydrocarbons, and in polymer degradation (Mendenhall, 1990).

2. Materials

Chromatographically purified soya phosphatidylcholine ($\geq 98\%$; batch no. E 40302) and medium-chain monoacylglycerol ($\geq 99\%$; approximately 20% C10:0 and 80% C8:0 fatty acids; batch no. K 40517) were obtained from Karlshamns LipidTeknik AB, Stockholm, Sweden. Samples for the oxidation studies were prepared by dissolving S-PC in molten MCM (melting point 32–35°C). Appropriate amounts of the components were weighed and sealed in a glass bottle under nitrogen. The mixture was stirred with a magnet and held between 50 and 60°C for 2–3 h as needed to obtain a uniform solution. (The heating time required depends on the percentage S-PC to be dissolved. Smaller proportions of S-PC can be used to avoid any uncertainty caused by the dissolution procedure).

The mixtures obtained above were divided into smaller glass vials for a storage study. Two sets of samples were stored at 40°C after having sealed the vials under dry nitrogen purge and in air respectively. An extra set of sample vials from the 50/50 mixture was sealed under dry nitrogen and stored at −20°C also. The 50/50 w/w S-PC/MCM samples were analyzed at 60, 204, 492, 708, and 972 h while the 30/70 w/w S-PC/MCM samples were analyzed after 254, 470, 686, and 854 h. A whole battery of analyses was made on each vial; two vials were analyzed at each point in time except for microcalorimetry and HPLC.

3. Methods of analysis

The following analyses were performed on the stored samples.

3.1. Thiobarbituric acid reacting substances (TBARS)

The TBARS assay of Buege and Aust (1978) was used in a modified form. Butylated hydroxytoluene was added to the reagent to prevent further oxidation during the heating step which was carried out in boiling water.

The presence of TBARS was determined by measuring absorbance at 532 nm against reagent solution as reference (Hitachi U2000 spectrophotometer). Known concentrations (0.16–1.60 μg/ml) of MDA (1,1,3,3 Tetramethoxy propane; Sigma T1642) were used as standards. Results presented here have been normalized with respect to initial S-PC content in the sample analyzed.

3.2. Iodometric assay

This method is based on the ability of I^- to reduce hydroperoxides (Buege and Aust, 1978). Under the conditions employed here, only hydroperoxides react with iodide, thus excluding the cyclicperoxides that form MDA from the assay. The molar extinction coefficient of cumene hydroperoxide (1.73×10^4/M/cm) was used to calculate the concentration of lipid hydroperoxides in the S-PC/MCM mixtures (absorbance measured at 353 nm). Results have been reported after normalizing with respect to initial S-PC content in the samples.

3.3. UV absorption 233 and 270 nm

UV absorption spectra of the lipid samples (approximately 20 mg) dissolved in ethanol (25 ml, spectroscopic quality) were measured. The reference cuvette contained the same amount of MCM (as in the samples) dissolved in ethanol (25 ml). Measurements were made on a Hitachi U 2000 spectrophotometer using 10 mm quartz cuvettes.

Klein (1970) used the ratio of absorbances ($A_{233\ nm}/A_{215\ nm}$) as an oxidation index. The rationale is that 215 nm is the lowest wavelength that depends linearly on the amount of material used but is far enough away from 233 nm to be free from interference due to oxidation. Konings (1984) reported that for oxidation indices higher than 0.8, the wavelength at 215 nm is not independent of the degree of oxidation. We examined this problem by mixing a relatively unoxidized 50/50 S-PC/MCM batch with an older oxidized batch (denoted as B) in various proportions to obtain samples with varying degrees of oxidation. UV absorption measurements were carried out as indicated above and the results are summarized in Fig. 1. The measured absolute absorbances at 233 and 270 nm were found to increase linearly with increase of proportion of batch B, while the oxidation index ($A_{233\ nm}/A_{215}$ nm) as defined by Klein (1970) changes slope at around 30% B, as does the ratio ($A_{270\ nm}/A_{215}$ nm). It was therefore decided to use absolute absorbances at 233 and 270 nm after correcting for any baseline shift caused by light scattering effects, i.e. ($A_{233\ nm}-A_{300\ nm}$) and ($A_{270\ nm}-A_{300}$ nm) as the oxidation indices in this study. This allows for a linear response to be obtained for a wider range of sample oxidation levels.

For the purpose of comparison of the 50/50 and 30/70 samples, the above defined absorbance differences have been recalculated to a reference weight of 0.02 g S-PC.

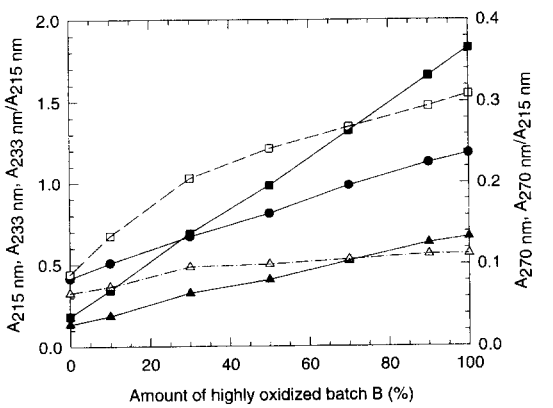


Fig. 1. Absolute UV absorbances are linear functions of extent of oxidation while oxidation indices are non-linear. Samples with increasing degrees of oxidation were obtained by mixing a fresh and an oxidized 50/50 S-PC/MCM batch B. (– ● –) $A_{215\ nm}$, (– ■ –) $A_{233\ nm}$, (– ▲ –) $A_{270\ nm}$, (– □ –) $A_{233\ nm}/A_{215\ nm}$, (– △ –) $A_{270\ nm}/A_{215\ nm}$.

3.4. HPLC with ELS-UV detection

A straight-phase HPLC method was developed to measure S-PC content and oxidation products in the samples. The system consisted of Shimadzu LC-6A pumps, Shimadzu system controller SCL-6A, WISP 717 plus autosampler and a Shimadzu C-R3A integrator. Detection was by an ELSD from S.E.D.E.R.E. (Sedex 45) followed by a UV detector (Spectromonitor III, LDC/Milton Roy). A Lichrosorb di-ol (100 × 4.6) 5 μ column from Merck was used with isocratic elution by a (propan-2-ol:*n*-heptane:water::57.7:38.8:3.5) mobile phase. The *n*-heptane was of extra pure quality from Merck and propan-2-ol was HPLC quality from Lab-Scan Analytical. The lipid sample was dissolved to a concentration of around 100 μg/ml in propan-2-ol. Injection volume was 20 μl and the flow rate 1 ml/min. The ELSD was run at a temperature of 40°C and a nebulization pressure of 2.6 bar.

The ELSD was used for concentration measurement (Hopia and Ollilainen, 1993) and the UV detector to monitor (at 230 nm) oxidation products. Of the three relevant peaks in the UV chromatogram (at 3.5, 4.1 and 5.0 min), the first is attributed to S-PC, the others to oxidation products; these products have not been identified or quantified absolutely. Instead two peak indices were defined to reflect extent of oxidation

Index 1 = (area peak # 2/area peak # 1), and
Index 2 = (area peak # 3/area peak # 1).

3.5. Isothermal microcalorimetry

We have used isothermal microcalorimetry to quantify the extent of oxidation already present in the sample. The S-PC/MCM sample (70–100 mg) was placed in glass ampoules which were filled with dry nitrogen and sealed. These ampoules were then inserted into the Thermal Activity Monitor® (Thermometric AB, Järfalla, Sweden) running at a temperature of 80°C. The samples were monitored for 72 h against an empty ampoule as reference. The total heat evolved over a period of 60 h, heat flux at peak maximum, and time to peak maximum were related to the extent of oxidation. The energies were normalized with respect to initial S-PC weight in sample analyzed.

Preliminary studies were made to confirm that the heat flux measured were indeed from oxidation. They showed that signals from lipid samples sealed in ampoules under nitrogen return slowly to baseline. The signal rebounds when the ampoule is removed, opened to air, resealed and placed back in the calorimeter. A microcalorimeter measures heat from all events occurring in the sample. Thus it can be deduced that the heat flux is due to the set of reactions indicated in Scheme 1 and other secondary reactions of oxidation. As the oxygen dissolved in the sample is consumed, the termination reactions take over and finally all oxidative activity stops. The heat flux thus returns to baseline. On re-aerating the sample, the process of oxidation gets restarted and therefore leads to a rebound of the signal.

3.6. Chemiluminescence

CL measurements were made with a CLD 100 chemiluminescence detector containing a CLC-10 photon counter (detection range 300–800 nm) from Tohoku Electronic Industrial Co., Miyagi, Japan. The apparatus has a compartment in which an open container containing the sample can be placed. The whole compartment can be heated; a shutter isolates the photomultiplier tube from this compartment when measurements are not being made. HPLC sample vials were cut to obtain small glass cups (Ø13 mm; height 10 mm) which were used to hold the lipid sample ($\sim$200 mg) in the CL apparatus. The cups with the samples were always introduced into the compartment when the compartment was at 26°C. Prior to starting measurements, the compartment was flushed with nitrogen for 10 min and the nitrogen flush was continued throughout the measurement period. At time $t = 0$, the nominal temperature setting of the compartment was raised to 100°C; the CL counting was started simultaneously. Counting was continued for 2500 s, in packets of 10 s after which the sample was removed and the compartment cooled to room temperature. The data was corrected for dark current intensity measured at the end of each run. Values are reported after normalizing the cumulative-counts with respect to unit weight S-PC in the sample analyzed.

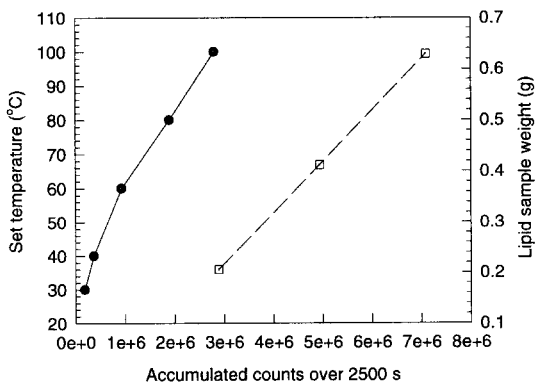


Fig. 2. Accumulated chemiluminescence counts are strongly dependent on the (– ● –) set temperature of the sample compartment. The cumulative counts are a linear function of the (– □ –) weight of the 50/50 S-PC/MCM sample in the cups.

In preliminary experiments, it was found that the glass cups themselves gave no CL signal when new and washed with soap and cold water. However, if the cups were washed in hot water or were preheated and cooled before use or were reused, a higher background CL signal could be detected from the glass itself; new cups were therefore used for each sample and run. Tests with different weights (200, 400, 600 mg) of the same lipid sample showed that total CL counts were directly proportional to sample weight. It is important to ensure that the geometry of the sample emitting photons is the same in all experiments. It was therefore decided to use a sample weight of approximately 200 mg; a minimum weight of 150 mg is required to cover the bottom of the cup. The temperature dependence of the total CL intensity was also investigated by making measurements at compartment temperature settings of 30, 40, 60, 80, and 100°C. The total counts increases linearly with temperature (Fig. 2). A setting of 100°C was chosen for the subsequent measurements since this temperature gives the highest counts and a sharper peak (data not shown). Furthermore, the initial temperature of the sample compartment was found to be critical for the reproducibility of the method.

It was also found that the compartment temperature shown on the apparatus did not correspond to the actual sample temperature during the heating-up period. The actual temperature profile was measured and found to be very reproducible when using identical operating conditions (Singh et al., 1996).

Other runs made with pure MCM (at 60°C) showed no CL signals from this component. The CL emanating from a mixture can thus be attributed to the S-PC.

4. Results and discussion

Results from each of the methods are presented here and then correlated with those from CL measurements.

4.1. Thiobarbituric acid reacting substances (TBARS)

The results from this method showed that the amount of MDA increases significantly (after 21 days) for the 50/50 S-PC/MCM sample held in air, while MDA remained constant for those held in nitrogen irrespective of temperature (Fig. 3). The increase in MDA for the 30/70 S-PC/MCM sample is somewhat lower (Fig. 3). The individual data points from each vial are shown in Fig. 3.

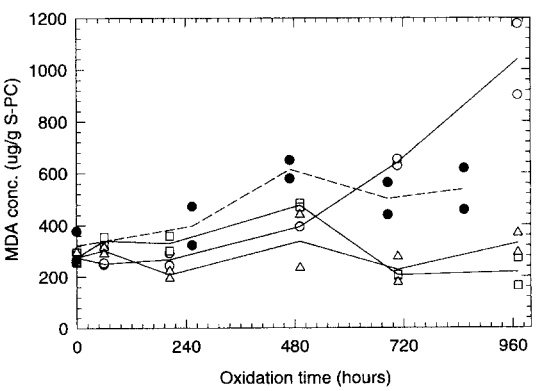


Fig. 3. Malondialdehyde concentration in lipid samples by the TBARS method, as a function of oxidation time for 50/50 S-PC/MCM (= open symbols) and 30/70 S-PC/MCM (= filled symbols) in (– ○ –, – ● –) 40°C in air; (– □ –) 40°C in N_2; and (– △ –) – 20°C in N_2.

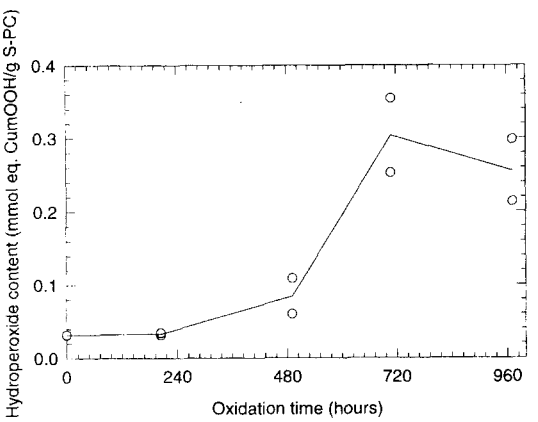


Fig. 4. Development of lipid hydroperoxides (as mmol equivalent cumene hydroperoxide) in 50/50 S-PC/MCM samples stored at 40°C in air measured by the iodometric assay.

4.2. Iodometric assay

The hydroperoxide content of the 50/50 S-PC/MCM sample held in air at 40°C is shown in Fig. 4. The concentration of hydroperoxides increases drastically after 21 days and appears to decrease slightly after 30 days. The individual data points from each vial are shown in Fig. 4.

Samples at higher oxidation levels show higher variability in the results from this method.

4.3. UV absorbance 233 and 270 nm

The conjugated dienes ($A_{233\ nm}-A_{300\ nm}$) and trienes ($A_{270\ nm}-A_{300\ nm}$) increase rapidly on storage in air after an initial lag period (Fig. 5(a, b)). The dienes have a shorter induction period of about 9 days although the concentration (of trienes too) increases much more rapidly after 21 days. Amount of trienes formed is much lower than dienes since S-PC has only 7 wt.% C18:3 fatty acids compared with 63 wt.% C18:2 fatty acids. Furthermore oxidization of the C18:3 fatty acids can first lead to the formation of conjugated dienes, which then are further oxidized to form trienes. This rearrangement of dienes to trienes could account for the decrease of ($A_{233\ nm}-A_{300}$ nm) after 30 days for the samples held in air.

The reproducibility of the data obtained by this method was very good, the results for two vials at any time point differing by 2–4% only. Therefore, only the mean values are shown in the figures.

4.4. HPLC with ELS-UV detection

Determination of unoxidized S-PC concentration shows that the 50/50 S-PC/MCM sample oxidizes more rapidly than the 30/70 material (Fig. 6(a)). The results of this analysis reported as Index 1 and 2 also clearly shows an induction period of about 21 days for the 50/50 sample held in air at 40°C as shown in Fig. 6(b, c). The value of these indices are however higher for 30/70 S-PC/MCM as compared with those for the 50/50 samples. This is in contrast to the results obtained

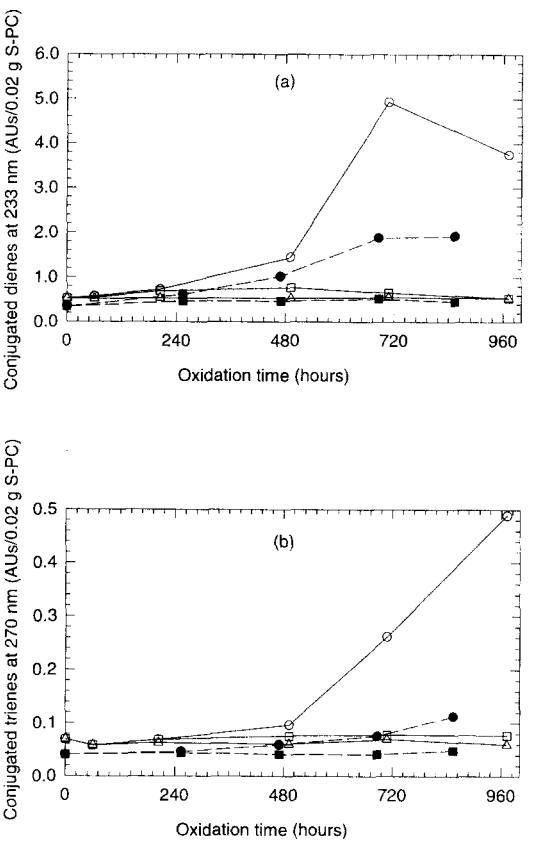


Fig. 5. UV absorbtion assay on the 50/50 S-PC/MCM (= open symbols) and 30/70 S-PC/MCM (= filled symbols) lipid samples stored at (– ○ –, – ● –) 40°C in air; (– □ –, – ■ –) 40°C in N_2; and (– △ –) – 20°C in N_2. (a) Conjugated dienes at 233 nm. (b) Conjugated trienes at 270 nm.

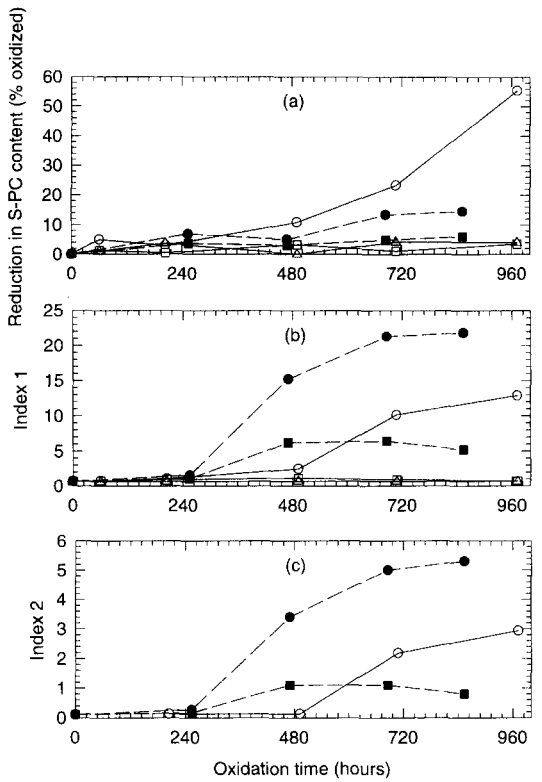


Fig. 6. Oxidation as measured by the HPLC-ELS-UV method on the 50/50 S-PC/MCM (= open symbols) and 30/70 S-PC/MCM (= filled symbols) lipid samples stored at (– ○ –, – ● –) 40°C in air; (– □ –, – ■ –) 40°C in N_2; and (– △ –) – 20°C in N_2. (a) Percent reduction in S-PC content. (b) Index 1. (c) Index 2.

by all the other methods. A probable reason for this observation could be that since the oxidation of the 50/50 S-PC/MCM material has proceeded further along the reaction cascade, the products that elute in peaks 2 and 3 are present to a lesser extent in this sample. This reduces the effective area of these peaks and thereby the index values.

4.5. Isothermal microcalorimetry

The lipid samples show exothermic activity when introduced into the microcalorimeter at 80°C. The signal rises rapidly and reaches a peak before returning slowly to the baseline over a period of about 50–60 h (see Fig. 7). On introduction into the apparatus, the temperature of the sample rises rapidly, increasing the rate of all reactions occurring in the sample. However, since the ampoules are devoid of oxygen, the oxidation cascade comes to a standstill and activity eventually returns to baseline.

The signal has been quantified by determining the time to peak maximum, the heat flux at peak maximum, and the total heat evolved over 60 h. There is a general trend towards decreasing time to peak maximum with increasing degree of oxidation (data not shown). The peak occurs due to increasing rate of reaction being countered by decreasing reactant concentration. If the rate of change of reaction rate is equal for all samples, the difference in peak positions indicates lower amounts of dissolved oxygen present in the more oxidized samples. The heat flux at peak maximum and total heat evolved also increases for the samples held at 40°C in air. An induction time of about 21 days is observed with this method too (Fig. 8(a, b)). The 50/50 S-PC/MCM samples again show a higher degree of oxidation as compared with the 30/70 S-PC/MCM samples.

Smaller increases in peak heat flux and total heat for samples held in nitrogen can be at-

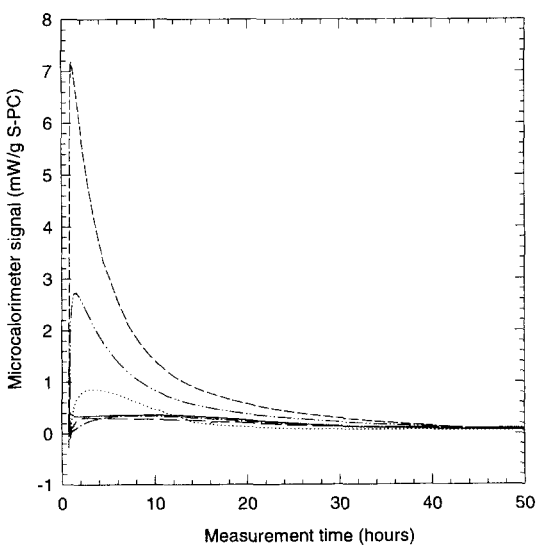


Fig. 7. Heat evolution during the measurement of oxidation by isothermal microcalorimetry on 50/50 S-PC/MCM lipid samples pre-oxidized in air at 40°C for (——) 0 h, (– –) 60 h, (• – •) 204 h, (···) 492 h, (• • – – • •) 708 h, (– – –) 972 h.

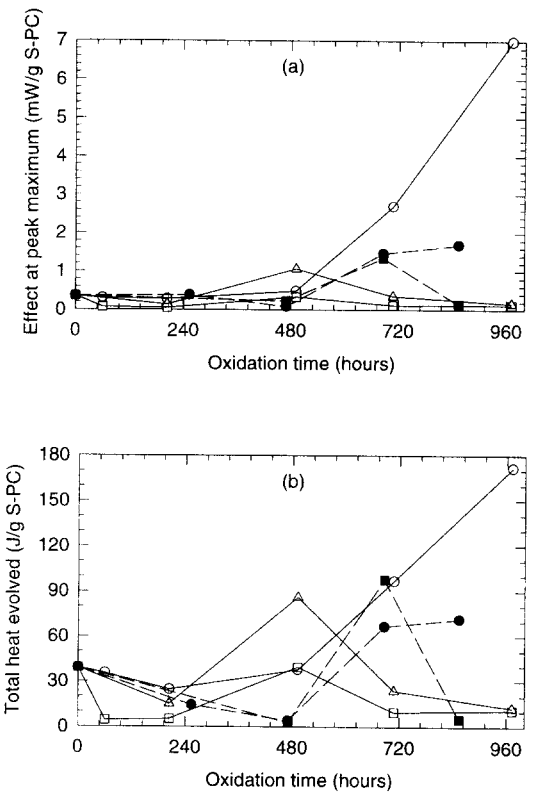


Fig. 8. Oxidation parameters from isothermal microcalorimetric analysis of 50/50 S-PC/MCM (= open symbols) and 30/70 S-PC/MCM (= filled symbols) lipid samples stored at (– ○ –, – ● –) 40°C in air; (– □ –, – ■ –) 40°C in N_2; and (– △ –) – 20°C in N_2. (a) Effect at signal peak maximum. (b) Total heat evolved over a 60 h measurement period.

tributed to the oxygen dissolved in the samples and any left in the vials before sealing. The seemingly random variation in the total heat evolved in these samples however shows some clear trends on a closer look (Fig. 8(b)). Samples at different stages in the oxidation process will show different degrees of thermal activity and the ability of the instrument to measure a sum of these activities is reflected in the data obtained. For samples stored under nitrogen, there occurs first a decrease, followed by an increase and then decrease again in the total heat evolved. This is seen for both compositions except that the higher MCM content slows the process for 30/70 samples. For the samples stored in air, the first part is the same; however, the second reduction in total heat evolved is replaced by a strong increase. If correlated with the actual oxidation reactions occurring at the different points in time, this could be used as a finger-print for the process. The thermal activity of the material held under nitrogen goes down to near zero after 30–40 days implying a termination of all reactions when the oxygen in the sample and vial has been consumed.

4.6. Chemiluminescence

A set of CL curves obtained for 50/50 S-PC/MCM samples stored in air at 40°C is shown in Fig. 9. As the degree of oxidation of the samples increases, the CL curves become broad and high with the peak appearing later during the measurement. Based on the studies made on polymer oxidation (Billingham et al., 1991) it can be speculated that the initial peak seen in the less oxidized samples is probably due to a reactive fraction of the total peroxides, possibly the linear hydroperoxides. As the oxidation proceeds, the content (proportion) of the secondary peroxides (e.g. the cyclic peroxides) in the sample increases. Decomposition of these secondary peroxides occurs by a multitude of pathways and could be the source of radicals that produce CL at higher temperatures; therefore the peak appears later in the measurement when the sample temperature is higher

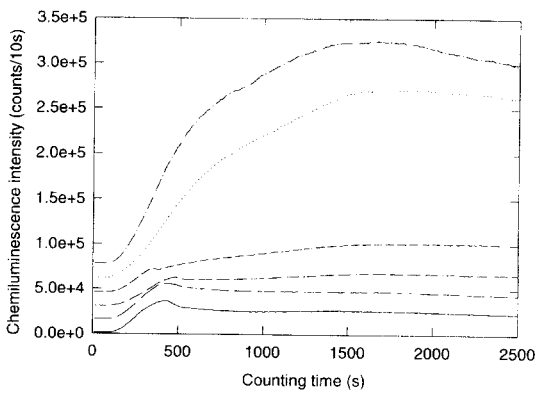


Fig. 9. Chemiluminescence intensity curves obtained during CL analysis of 50/50 S-PC/MCM lipid sample pre-oxidized in air at 40°C for (——) 0 h, (— — — —) 60 h, (– –) 204 h, (- - -) 492 h, (···) 708 h, (• – •) 972 h. The curves have been successively shifted upwards by 15 000 counts each for clarity.

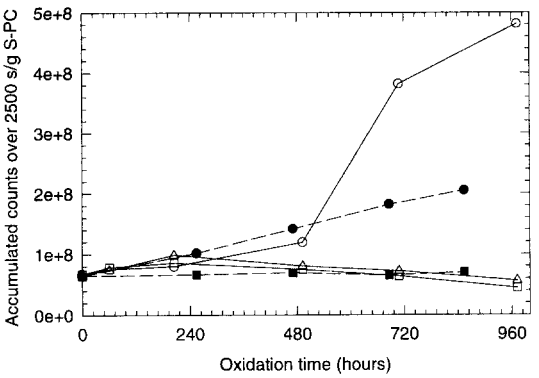


Fig. 10. Accumulated chemiluminescence counts over 2500s from CL analysis of 50/50 S-PC/MCM (= open symbols) and 30/70 S-PC/MCM (= filled symbols) lipid samples stored at (– ○ –, – ● –) 40°C in air; (– □ –, – ■ –) 40°C in N_2; and (– △ –) – 20°C in N_2.

(Singh et al., 1996). This effect is more apparent in material with higher degree of oxidation. Singh et al. (1996) also indicate a possible shift in the mechanism from a bimolecular to a unimolecular reaction for the emission of CL during measurements.

The accumulated counts over the measurement period of 2500 s are shown in Fig. 10 as a function of oxidation time. Mean of counts from two vials are plotted; variability between two vials was always less than 5%, with most of the samples being under 2%. The sample stored in air at 40°C again shows a large increase in accumulated CL counts after an apparent induction period of 21 days. This trend is stronger in the 50/50 S-PC/MCM sample implying that the degree of protection provided by MCM is in proportion to its concentration. Smaller peaks in the total CL counts for the other samples around 10 days (Fig. 10) can be attributed to oxygen dissolved in sample or trapped in the vial despite the nitrogen purge.

4.7. Correlation between chemiluminescence and other methods

A comparison of the methods is made in Fig. 11(a–d) for the 50/50 S-PC/MCM sample stored in air. The plots can be clearly divided into two parts: a low oxidation level range prior to 30 days and high oxidation levels thereafter. Linear regression has been performed on all but the last point (from 0–30 days; solid lines in the plots). Results show that the TBARS method does not correlate well with the accumulated CL counts (Fig. 11(a)). This is reasonable considering that CL detects hydroperoxides and TBARS measures one product (of many) from decomposition of the hydroperoxides.

In contrast, the relation between CL and peroxide value is linear up to 30 days. The iodometric method measures only the linear hydroperoxides which are directly reflected in the CL value. However, as the proportion of the secondary peroxides increases as discussed earlier, the slope of the plot in Fig. 11(b) changes (also compare Figs. 4 and 10).

The absorbance from conjugated dienes, 233 nm, and trienes at 270 nm almost parallels the development of the CL signal; CL detects peroxides formed from both conjugated dienes and trienes and therefore mirrors a sum of these (Fig. 11(c); compare Fig. 5(a, b) with Fig. 10).

Of the results obtained from microcalorimetry, the heat flow at peak maximum shows good correlation with CL (Fig. 11(d)). However, after 30 days, the CL curve changes slope while the microcalorimetry signal keeps increasing since other reactions (than those that generate CL) also contribute to the heat effect. Correlation with the HPLC method is not shown but it is apparent from comparing Fig. 6(a, b) with Fig. 10 that the trends are similar.

R^2 values obtained for the linear regression (on points upto and including 30 days, i.e. all but the last point) are 0.954 for TBARS and better than 0.990 for the other parameters. The discontinuity between the first and second points in all the measurements reduces the correlation coefficient; this discontinuity can be attributed to oxidation induced during the sample preparation and dispensing procedure.

A general comparison of the methods used here shows that only the TBARS, UV abs. 270 nm, microcalorimetry and CL measurements return monotonically increasing values with increasing extents of oxidation. It must however be noted that conjugated trienes will be formed in only

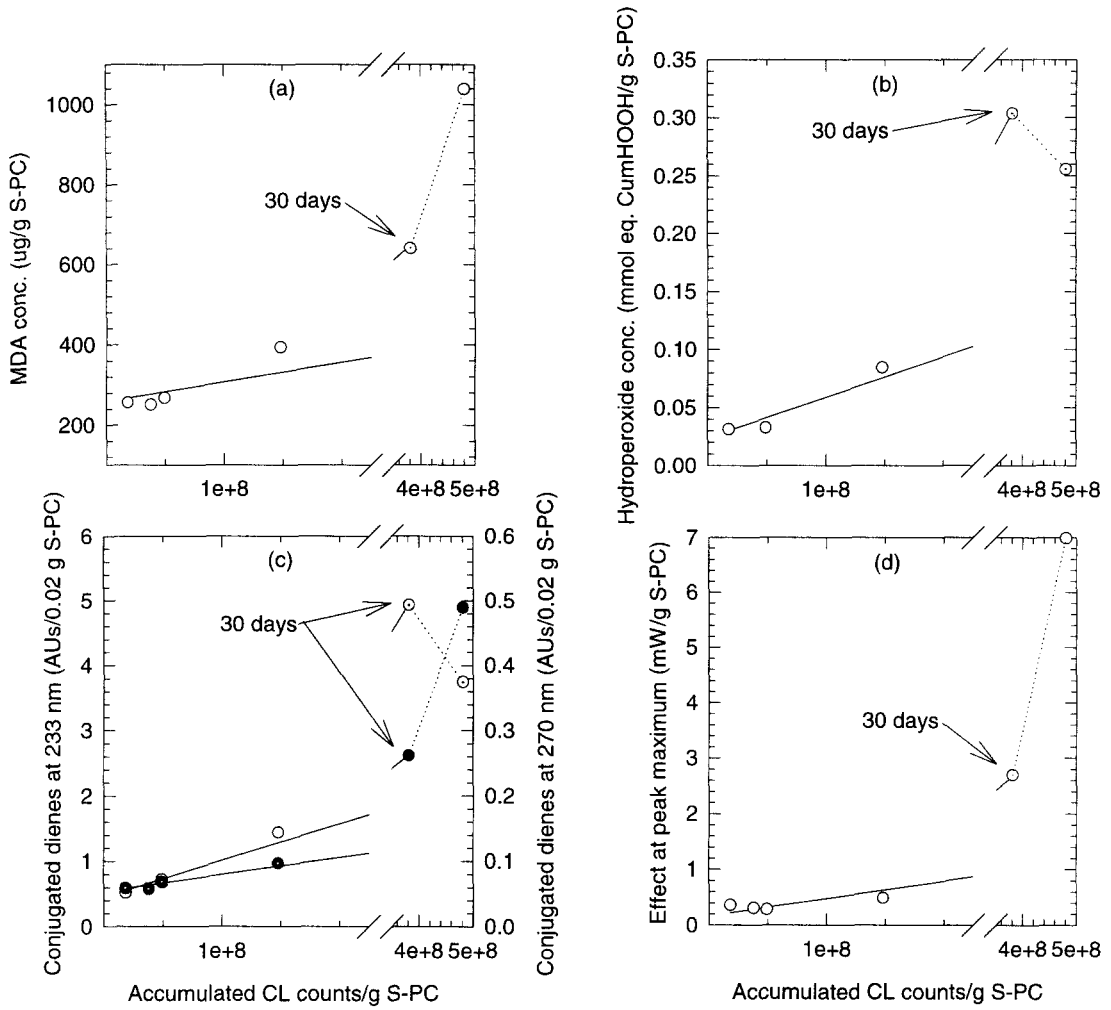


Fig. 11. Correlation between chemiluminescence and other methods for assesment of oxidation of 50/50 w/w S-PC/MCM lipid samples stored in air at 40°C. The solid line has been obtained from linear regression over data from 0–708 h, by including all but the last data point. (a) Malondialdehyde concentration by the TBARS method vs. accumulated CL counts. (b) Hydroperoxide concentration as mmolar equivalent cumene hydroperoxide measured by the iodometric assay vs. CL counts. (c) Conjugated dienes (– ○ –) at 233 nm and conjugated trienes (– ● –) at 270 nm vs CL counts. (d) Effect at signal peak maximum during isothermal microcalorimetry analysis vs. CL counts.

those phospholipids that have triple-unsaturated fatty acids. The iodometric assay peroxide value, and UV abs. 233 nm values decrease at higher extents of oxidation which can be misleading. This disadvantage is also shared by the indices defined for the HPLC-ELS-UV method. However, as oxidation proceeds even further, it is probably only the microcalorimeter that will continue to return increasing 'extent of oxidation' values.

In conclusion it can be said that CL can be used as a convenient and sensitive method to measure oxidation status of phospholipids. It can serve as a good complement to the conventional methods, and can be especially useful as a screening tool during developmental work.

4.8. Chemiluminescence method development

A note must be added as to the physical nature of the sample that can be analyzed by the CL technique. The intensity of CL will be proportional to sample area exposed. For transparent or translucent samples, the CL will be proportional to sample mass provided the depth/thickness of the sample is low. For opaque materials, the CL will be only proportional to area exposed. Thus, for phospholipid raw materials or microparticulate formulations, the procedure would involve a dissolution step, either into MCM as in this report, or some other suitable solvent. Freeze dried formulations containing lipids may be preferably analyzed by reconstituting and analyzing an aliquot even though the lyophilized cake may be analyzed directly. A suitable test cell need be designed in those cases where a solvent or a solution is present; however, note that the cell cannot be made of a polymeric material due to the tendency of these materials to emit CL themselves.

5. Conclusions

Two new methods for measuring phospholipid oxidation have been developed and tested here. This is the first report on the use of isothermal microcalorimetry and direct chemiluminescence measurement for soya phosphatidylcholine oxidation; monoacylglycerol has been used as a novel solvent. Both these techniques show good sensitivity to the degree of oxidation the samples have undergone. Chemiluminescence is however rapid and easier to measure and shows good correlation to the iodometry and UV absorbance based methods; correlation with TBARS is weaker. The correlation or lack thereof can be explained on the basis of the mechanism of phospholipid oxidation. Both CL and microcalorimetry return values which increase monotonically with increasing oxidation.

Use of MCM as solvent makes S-PC samples easier to handle. MCM also serves to protect S-PC from oxidation, probably by reducing the area of exposure (freeze-dried S-PC is highly hygroscopic with a crumbly particulate appearance).

The methods developed above should be useful in assaying lipid raw material, and (phospho)lipid-based preparations, e.g. freeze-dried liposomes, matrices, microparticles, lipoproteins, suspensions, ointments etc. Some practical aspects of method development have been discussed.

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